BACTERIAL SPECIES OF THE RUMEN

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I. INTRODUCTION

The ruminant differs from other mammals in that its food is subjected to microbial fermentation in the rumen before it passes on to the true stomach and intestinal tract where normal mammalian digestion occurs. Processes taking place in the rumen due to microbial activity include the degradation of carbohydrates such as cellulose that cannot be utilized unless digested by microorganisms and those such as starch and certain sugars that can be utilized by the animal without microbial action. Proteins, organic acids, and many other feed constituents are also attacked. The principal products are volatile fatty acids, carbon dioxide, methane, ammonia, and microbial cells. The fatty acids and many constituents of the microbial cells

such as vitamins and protein are utilized by the animal. It is known that, in addition to making constituents of the food available for the animal, synthetic processes such as the vitamin and protein production by the microflora may assume considerable importance. The ruminal fermentation has a considerable effect on metabolic processes of the animal and the functions of the microorganisms are intimately associated with certain metabolic disorders of ruminants. It has been increasingly realized that more fundamental knowledge of the ruminal microorganisms is needed to obtain more efficient rations and better control of metabolic disorders. Also, the rumen is a natural microbial habitat that has a far more constant environment and appears to be more susceptible to analysis by microbial ecologists than many other microbial habitats (44).

The environment of the rumen is well adapted for the maintenance of a large and diverse microbial population. There is a relatively constant supply of food and water. The temperature is held relatively constant at about 39 C. The pH of the ingesta, usually slightly acid, is held relatively constant by the influx of food, water, and heavily buffered saliva and the apparent tendency toward an equilibrium between the ruminal ingesta and the blood stream with regard to H ions (99). There is a constant removal of the products of microbial growth via secondary fermentations, passage to the lower digestive tract, and absorption through the rumen wall into the blood stream. The E_h of the ingesta is held at a low level due to the intense microbial activity and the low oxygen tension of the gaseous phase.

Since the 1940's, when British workers (see 33) emphasized the importance of volatile fatty acids produced by microorganisms in ruminant nutrition and Hungate (71, 72), Sijpesteijn (118, 119), and Gall *et al.* (41) published on methods of cultivating authentic ruminal bacteria, a rapidly expanding amount of information has accumulated on the ruminal fermentation. As our knowledge increases it is evident that it is gained by the use of many different techniques by workers of diverse training (25). It is also evident that the study of individual representatives of the ruminal flora and fauna by bacteriologists and protozoologists has contributed substantially to this knowledge.

Many different groups of microorganisms have been isolated from the rumen. However, it is now generally believed that organisms of functional significance in the rumen are the protozoa and bacteria capable of growth under the anaerobic conditions prevailing. The work on ciliate protozoa has been recently reviewed by Oxford (106) and Hungate (73). Some of the more recent reviews on rumen bacteriology include those of Doetsch and Robinson (27), Oxford (107), and Briggs (8). Reviews of the earlier work on cellulolytic bacteria are available (72, 118). This review is limited to pure culture studies with emphasis on the species of bacteria cultured.

II. THE CULTURE OF RUMINAL BACTERIA

A. Authentic Ruminal Bacteria

While detailed studies on the growth and metabolism of any species of bacteria isolated from the rumen might be beneficial to basic knowledge of bacteriology and comparative biochemistry, it is evident that in attempting to apply this knowledge to the ruminal fermentation, the species studied should be those functional in the rumen. It is rather common knowledge that many species of bacteria present in the rumen are not functional but are merely casual passengers brought in with the food (see Sijpesteijn (118) and Hastings (57)). Also, it has been suggested that in young animals, organisms functional in the abomasum may gain entrance to the rumen (95). For the most part, these species account for only a minute part of those revealed by the microscope. However, some instances are known where very large numbers of bacteria may be ingested with the feed (48). Many workers have discussed criteria to be used in determining an organism's significance in the rumen (see Elsden and Phillipson (33), Gall and Huhtanen (40), and Briggs (8)). Yet work continues to appear on various characteristics of isolates where little or no evidence is obtained on the organisms' significance.

Probably the most important criteria that can be used are that the organism be shown to grow in the rumen and that it have a metabolism compatible with the reactions occurring and the environment present in the rumen. The numbers of a given species present in the rumen as compared to its numbers in the feed and water consumed, along with similar data on other species carrying out the same reactions, are probably the best criteria for use at the present time. The numerical criterion alone can establish that an organism is growing in the rumen and, therefore, that the protoplasm synthesized is available to the host.

While other criteria are of relatively little value

in the absence of the numerical criterion the reverse is also true. The organism should be able to attack substrates present in the rumen. These are not necessarily materials present in the feed but may include hydrolytic products of feed constituents such as cellulose, starch, and protein, and fermentation products such as organic acid and hydrogen. It is also possible that considerable amounts of substrate are available in the form of slime and cellular constituents of organisms that die in the rumen. Fermentation products should be compatible with those present in the rumen or metabolized therein. However, some species of rumen bacteria, which present evidence indicates to be of considerable significance, produce *in vitro* large amounts of ethanol (18, 21), which does not appear to be present or metabolized in the rumen under usual conditions. The production of ethanol by these pure cultures is probably due to the artificial environment and a study of factors affecting its production might lead to new knowledge of interactions occurring in the rumen. Cultural characteristics such as E_h, pH, and temperature range of growth of the pure culture as well as nutritional requirements should be compatible with the environment present in the rumen. Also, the rumen may contain substances that inhibit the growth of species that might otherwise be able to function (42, 65).

It is evident that many criteria might be used in obtaining evidence of an organism's significance and that the individual worker may not be able to apply many of these. In the final analysis, knowledge of the relative significance of an organism will be gained through the application of many different criteria by workers using various approaches.

B. Sampling Rumen Contents

Methods of obtaining and processing samples of rumen contents for the culture of bacteria have received relatively little attention and in many studies the choice of method is not of particular importance. However, in studies concerning comparisons of numbers and kinds of bacteria present in the rumen under various conditions, the sampling method might be of considerable significance. Rumen content is a heterogeneous mixture with various degrees of stratification depending on such factors as the time after feeding, the type of ration, and the water supply. There is abundant evidence that samples taken from different locations in the rumen vary considerably in chemical composition and in rates at which various reactions occur (121). Also, the reproducibility of data on rates of reactions can vary considerably depending on such factors as the time the sample is taken and water supply (76).

By use of direct microscopic methods, it has been shown that numbers of bacteria vary somewhat through the day in animals on a constant ration and the trend in numbers is affected by the method of feeding, such as number of feedings and whether concentrates and roughage are fed separately or together (101, 103).

Very few studies of sampling methods have been made that involved cultural methods. Hungate (71) obtained roughly equal numbers of cellulolytic bacteria in liquid and solid samples. Gall *et al.* (41) obtained higher total cultural counts from the more solid samples and found counts from liquid obtained by stomach tube to be lower than from liquid obtained through a fistula. Fulghum *et al.* (38) found both proteolytic and total cultural counts to be slightly higher in samples taken from the bottom than from the top of the rumen. Higginbottom and Wheater (60) found that numbers of *Streptococcus bovis* varied 2- to 3-fold with time after feeding when cattle were stall-fed but varied little when cattle were on pasture. Wilson and Briggs (129) have studied various factors involved in sampling.

In comparing numbers and kinds of bacteria in the rumen under various conditions, most workers have sampled at a standard time during the day and, when animals with fistulas or slaughtered animals were used, from a certain location in the rumen. When samples are taken by stomach tube, the location sampled is often in doubt. If a relatively rigid, curved tube is used, it is possible to manipulate the end of the tube so that different samples are probably obtained from about the same location (113).

When animals with fistulas are available, enrichments of certain types of bacteria can be made by placing insoluble substrates in containers permeable to bacteria and suspending them in the rumen until the substrate is partially disintegrated. This is then removed and cultured. Van der Wath (124) used this sampling technique to isolate starch-grain-digesting bacteria.

It is evident that little is known concerning variations in numbers and kinds of bacteria due to time of sampling, location sampled, or feeding schedule of animals on a given ration. Some of the available information has been obtained with very crude culture-counting techniques that are inher-

ently more variable than the actual variability one might expect in the samples.

The method of processing the sample prior to inoculating culture medium can assume considerable importance. The importance of speed and exclusion of air from the sample have been emphasized (39). However, others noted that the reducing capacity of rumen contents is great and found no difference in total culture counts when rumen contents were exposed to air and held for considerable lengths of time before processing (28, 129). It may be that although changes in total counts are not evident qualitative changes are involved (129). It seems best that samples be processed as rapidly as possible, especially where one is attempting to correlate various quantitative data on the flora. However, it may be that some workers have sacrificed the accuracy of processing methods to too great an extent in order to decrease the time between obtaining the sample and completing inoculations.

Various workers have determined differences in bacterial counts depending on whether the whole sample or the more solid or liquid fractions obtained by squeezing the sample through cheese cloth were cultured. Huhtanen *et al.* (70) cultured the solid portion when stomach tube samples were used because it was believed that this overcame quantitative differences due to obtaining samples from different locations in the rumen where the contents may contain different amounts of dry matter. Some workers (12, 38) found higher total counts from the solid or whole contents than from the liquid portion but counts of proteolytic bacteria were the same (38). This suggests that the relative proportions of different groups of bacteria cultured can be changed by filtering to remove the larger feed particles.

Some workers have serially diluted the sample directly in tubes of culture medium. Others have used various dilution solutions and still others have used combinations of the two methods. The method of serially diluting the sample directly in the culture medium is satisfactory for many purposes but has disadvantages in that it is often difficult to obtain good dispersion of cells. Shaking media exposed to air tends to lower their reducing capacity and in agar media gas bubbles accumulate that are difficult to break down before the agar gels. Comparisons of subsamples serially diluted in media or in special anaerobic dilution solutions resulted in higher and less variable counts with the dilution solution in one study (12) and lower counts in another (129). However, substantially different solutions and media were used. It is evident that high counts and a wide variety of different species can be cultured when suitable diluents are used.

It has been shown that the type of diluent used may have considerable effect on the numbers of bacteria cultured. Gall *et al.* (41) used at first a 1 per cent glucose solution heated to drive off oxygen and cooled just before use, then changed to distilled water equilibrated with CO_2 or N_2 (39). Later, much better results were obtained with an anaerobic solution containing carbonic acid-bicarbonate and phosphate buffers with cysteine as reducing agent (70). Other workers have used similar anaerobic diluents but with more complex mineral mixtures. Resazurin has been included as an indication of anaerobiosis (12, 28); Tween 80 (Atlas Powder Company) (28, 129) to obtain better dispersion of cells; both cysteine and thioglycolate (28), or sulfide (129) as reducing agents; or no reducing agent (74). Data on the effects of various substances in the dilution solution are limited. Bryant and Burkey (12) found that distilled water equilibrated with CO_2 drastically lowered colony counts when compared to a complex mineral-cysteine solution with a carbonic acid-bicarbonate buffer, whereas only slightly lower counts were obtained when the latter solution was made aerobic by deletion of cysteine and CO_2 and replacement of sodium bicarbonate with sodium chloride. However, this solution when used as inoculum tended to oxidize the culture medium. Other data on the effect of aerobic conditions on viability of dilutions of anaerobic rumen bacteria are scarce. However, using dilute inocula in nutritional studies, Gill and King (45) found that an increase in the lag phase of growth of a strain of *Butyrivibrio* occurred when cysteine was deleted from the dilution fluid although heavy inocula in resazurin-oxidized media produced good growth after cysteine was added 27 hr later. *Bacteroides amylogenes* survived 5 days in the presence of O_2 (26). Bryant and Robinson (1958, *unpublished data*) found that deletion of cysteine from the dilution fluid used in preparing dilute inocula of *Bacteroides succinogenes* had little effect on cells centrifuged once and diluted but caused erratic results and greatly delayed growth in cells centrifuged twice. No comparisons have been made on the effect of kinds or concentration of ions present in the dilution fluid.

It seems probable that the best dilution fluid for rumen bacteria in general is one that has a moderate ionic concentration, has a buffer system holding

the pH at about 6.5 to 7.0, and maintains a relatively low E_h.

Microscopic examination of rumen contents reveals that many of the bacteria are not freely dispersed in the liquid but may occur in large clumps, in long chains, and in aggregates attached to the structural materials of the ingesta. Baker and Harriss in a series of direct microscopic studies distinguished between the fixed and the free bacterial population (4). Quite early in the development of cultural methods it was recognized that counts might be affected considerably depending on the degree of disruption of aggregates of bacteria. Hungate (71) obtained about the same counts of cellulolytic bacteria whether or not the undiluted rumen contents were mixed under CO_2 in a Waring Blendor for various intervals of time. Gall *et al.* (39) and many later workers have prepared a 1:10 dilution of the sample and mixed this on a mechanical shaker before further diluting the sample. No data are available on the effect of various degrees and times of shaking on the cultural count. A comparison of mixing 1:10 dilutions vigorously by hand or in a Waring Blendor indicated a higher count for the latter (12). However, microscopic observation showed that many large clumps of bacteria remained. The use of Tween 80 in dilution solutions to aid in dispersion of bacteria was referred to above.

C. Culture Media

Diverse culture media have been utilized for the isolation and/or enumeration of ruminal bacteria. Several workers have used indirect enrichment techniques that involve the inoculation of relatively large quantities of rumen contents into liquid media in which an attempt is usually made to approximate conditions in the rumen. The substrate of interest is added in an attempt to enrich that fraction of the flora that attacks the substrate. After growth becomes vigorous, isolation of the enriched flora is attempted using dilution or streaking methods with agar or liquid media containing the desired substrate. Most of the early work in attempts to isolate bacteria concerned with cellulose digestion in the rumen involved enrichment techniques and, although numerous cellulolytic bacteria were grown, none of them was later shown to be of significance in the rumen (72, 118). It appears that these techniques often select out bacteria that are present in small numbers and probably are not of functional significance in the rumen. In the few cases where it appears that important ruminal bacteria have been isolated by enrichment techniques involving large inocula, organisms with quite typical morphologies have been isolated and the cultural procedure has been followed quite closely with microscopic studies. Examples of these are the original isolation of *Peptostreptococcus elsdenii* (50) by Elsden *et al.* (34) and of iodophilic, amylolytic streptococci by Van der Wath (124). In the latter case, enrichment was made directly in the rumen as indicated above. In both of these cases, several workers later isolated the same species using direct methods of isolation in which a numerical estimate of the organism's significance in the rumen was obtained.

In most cases where authentic ruminal bacteria have been isolated, high dilutions of rumen contents have been inoculated directly into an agar medium for isolation of colonies or into liquid medium and then into agar for purification. The more direct method of using solid media has several advantages over liquid media. Less time and materials are involved in obtaining pure cultures and many different kinds of bacteria can be isolated from one sample of contents. When liquid media are used, one or a few types of bacteria will outgrow others so that only a few kinds can be isolated from one series of cultures. Also, in obtaining estimates of numbers of viable bacteria from one sample, an extremely large number of tubes of medium must be used as compared to colony counting methods with solid media (132). It has been shown by the use of direct microscopic counts that total numbers of bacteria per ml of rumen fluid, even under quite varied conditions, do not vary more than about 3-fold (101). Cultural counts involving inoculation of single tubes of liquid media with each of several 10-fold dilutions commonly show 10- and 100- and sometimes 1000-fold differences even when samples are obtained under a single standard set of conditions (70, 129). The use of appropriate colony counting techniques seldom shows more than 2-fold variations in samples obtained under a standard set of conditions (13, 28). It is possible that some species of rumen bacteria will not form colonies in appropriate agar media but no case is known where this is true.

The media utilized for primary culture of ruminal bacteria can be divided into nonselective media evolved to allow growth of the largest numbers of bacteria and the most diverse types, and media that are more or less selective for certain physiological groups.

For the most part two general types of nonselec-

tive media have been used. One type involves the use of sources of growth factors and nitrogen such as yeast extract and various types of protein hydrolyzates that are relatively reproducible and commonly used in bacteriological media. These usually are prepared as liquid media for primary culture followed by agar plates or shake tubes for purification.

Gall *et al.* (41) used a medium containing tryptone, peptone, beef extract, yeast extract, glucose, skim milk, phosphate buffer, and cotton cellulose. Later, Huhtanen *et al.* (70) deleted the milk, used lower concentrations of phosphate and glucose, and added cysteine and bicarbonate. Bauman and Foster (5) used a similar trypticase-phytone-glucose medium but minerals and a small amount of agar were added and no bicarbonate was used. Wilson and Briggs (129) used a slightly modified "reinforced clostridial medium" (61). This medium was similar to that of Huhtanen *et al.* (70) except that acetate and soluble starch, a small amount of agar, and no cotton were added and it contained a larger amount of bicarbonate and was equilibrated with carbon dioxide.

The other general type of nonselective medium includes modifications of the agar medium and anaerobic technique used by Hungate (71, 72) for the isolation of cellulolytic bacteria. Hungate (71) showed that the cellulose medium supported larger numbers of noncellulolytic than of cellulolytic bacteria. The success of the medium seemed to be that it was an attempt to approximate conditions in the rumen. It contained a mineral mixture; rumen fluid as a source of growth factors; a carbonic acid-bicarbonate buffer; and usually, cysteine as reducing agent and cellulose as substrate. Doetsch *et al.* (28) and Bryant and Burkey (12) used similar media but with glucose and cellobiose in place of cellulose as substrate.

Few comparisons of the two general methods of cultivating ruminal bacteria have been made. Indeed, it is difficult to compare data obtained by the different workers because with the Hungate medium, colony counts have been reported; and with the other, the highest dilution of rumen contents supporting growth in liquid medium is reported. Also, various differences in sampling and dilution techniques have been used that would affect counts. Some workers preferred the Hungate type medium to that of Gall *et al.* (41) but few comparisons were made (12, 28). King and Smith (82) compared total counts, cellulolytic counts, diversity of cellulolytic types, and cross inoculations of strains of cellulolytic bacteria using the Hungate type medium and that of Huhtanen *et al.* (70) modified to contain agar. The former was superior in all respects.

Several workers have compared colony counts obtained with a Hungate type basal medium with rumen fluid and with rumen fluid replaced or supplemented with various other sources of growth factors. Doetsch *et al.* (28) found that rumen fluid resulted in higher counts than ingredients of Eugonagar. Bryant *et al.* (20) compared similar media and obtained higher counts in the rumen fluid medium from rumen contents cultured from young calves as well as from adult cattle. Also, it was found that all groups of bacteria isolated from the trypticase-phytone medium were also isolated from the rumen-fluid medium but several groups isolated from rumen-fluid medium were not found in the other. McNeill *et al.* (100) compared singly and in mixtures various extracts and protein hydrolyzates commonly used in the cultivation of nutritionally extracting bacteria with rumen fluid and found much higher counts as well as more diverse morphological types of bacteria in the rumen-fluid medium. Also, when these substances were combined with rumen fluid, the counts were depressed as compared with rumen fluid alone. This suggested that many of the extracts and protein hydrolyzates contained substances inhibitory to rumen bacteria. Gilroy (46), in a study of nitrogen sources, came to the same conclusions using similar techniques. Similar conclusions have been drawn from data on pure cultures of ruminal cellulolytic anaerobes (1, 15). However, Maki and Foster (92) found that the inclusion of trypticase and phytone in rumen-fluid medium consistently increased counts above those obtained with rumen fluid alone. These workers and Wegner and Foster (127) found that total counts obtained when a basal medium containing trypticase and phytone was supplemented with rumen fluid depended on the ration fed to the animal. Higher counts were obtained in the supplemented medium when animals were fed high-roughage rations but counts were equal when the ration was mainly concentrate. The latter workers found that addition of a mixture of certain volatile fatty acids to the basal medium resulted in counts equal to those obtained when rumen fluid was added. This finding merits more study as it indicated that a medium devoid of rumen fluid and containing well standardized ingredients might be used to enumerate and isolate as diverse

a flora as obtained with the rumen-fluid medium. It is well known that rumen fluid included in culture medium varies somewhat from time to time even when collected from one animal on one ration and at a standard time after feeding. Wilson and Briggs (129) obtained no increase in total counts when rumen fluid was added to "reinforced clostridial" medium. However, no data were given and the method of obtaining counts was such that differences obtained by other workers would probably not be detected.

While the data available are conflicting, it seems advisable to include rumen fluid in nonselective media used for the isolation and enumeration of predominant ruminal bacteria until more complete information is available on their nutrient requirements. It should also be noted that growth requirements of bacterial strains may be more exacting on primary isolation than later when they have been grown in artificial media. Gilroy (46) noted this phenomenon with many strains of rumen bacteria.

Several lines of evidence indicate that high levels of bicarbonate and/or CO_2 are important in the isolation and enumeration of many rumen bacteria. Hungate (71, 72) and Sijpesteijn (118, 119) failed to obtain consistently good growth of cellulolytic bacteria until media were modified to contain large amounts of bicarbonate and CO_2. Huhtanen *et al.* (68, 70) obtained higher and more consistent counts and a more diverse flora when bicarbonate was incorporated into the nonselective medium of Gall *et al.* (41). Bryant and Burkey (12) showed that addition of bicarbonate was essential before growth of several groups of anaerobes was initiated in a complex basal medium and some groups produced a lower final pH in media with quite high levels of bicarbonate even though these media were much more highly buffered. Other work showed that some of these strains belonged to seven widely differing groups, namely, *Bacteroides succinogenes* (15), *Borrelia* (10), *Ruminococcus* (21), *Succinivibrio* (18), *Succinimonas* and *Bacteroides ruminicola* (19). Other rumen bacteria that appear to require or are greatly stimulated by bicarbonate and/or CO_2 in complex media are *Bacteroides amylogenes* (26) and *Bacteroides amylophilus* (54). A strain of *Butyrivibrio* required bicarbonate in a chemically defined medium and none of several organic compounds tested would replace it (45). Analysis of fermentation products of several of the succinic acid-producing species have shown a gross uptake of CO_2 (5, 22, 66, 78, 79) and Huhtanen *et al.* (67) studied the uptake of C^{14} from bicarbonate into the protoplasm or fermentation acids of certain rumen bacteria grown in complex media.

Energy sources, usually carbohydrate, are generally added to media for the "nonselective" isolation and enumeration of rumen bacteria. Glucose only (5), glucose and soluble starch (129), glucose and finely divided cotton (39, 41, 70), and glucose and cellobiose (12, 28, 82, 92) have been utilized for this purpose. Also, finely ground ruminant feeds and extracts of feeds such as hay or grain mixtures have been used (50, 74). No critical work has been reported comparing various concentrations and kinds of carbon sources to determine those allowing good growth of the largest numbers or of the most diverse kinds of bacteria. Wilson and Briggs (129) found no differences in total counts when cellobiose was added to their glucose-starch medium. However, it is known that a significant number of the predominant bacteria cultured in rumen fluid-glucose-cellobiose medium utilize cellobiose but not glucose for growth (12). These include many strains of cellulolytic ruminococci (21, 74, 119). Also, some strains of rumen bacteria utilize starch or maltose but not glucose or many other carbohydrates (54, 68), and xylan but not xylose (21), whereas others utilize organic acids or organic nitrogen compounds but not carbohydrates as energy sources (81). Hydrogen is probably the best energy source for ruminal methane bacteria (120). It seems probable that when isolations are made from media containing other energy sources, strains will be found that will not grow on the sources listed above.

One of the most important considerations in the isolation of predominant ruminal bacteria is the maintenance of a low oxidation-reduction potential in the medium. The principal methods used to exclude oxygen from the medium include the use of anaerobic jars (34, 81, 130), tubes of medium kept anaerobic by addition of a cap of "vaspar" (41, 70) or liquid paraffin (129), and the use of rubber-stoppered roll tube cultures with an oxygen-free gaseous phase, usually CO_2 or mixtures of CO_2 with N_2 or H_2. Many workers have used various modifications of the latter technique originally described by Hungate (72) for the isolation of cellulolytic anaerobes.

Most workers have added an oxidation-reduction indicator to culture media as a control to be sure that a low E_h is maintained. The most com-

monly used indicator is resazurin. Although this is a valuable indicator, certain observations indicate that media in which the resazurin is reduced to its colorless form may not be poised at an E_h sufficiently low to allow the initiation of growth or to minimize lag in the growth of certain ruminal anaerobes. Smith and Hungate (120) found it necessary to maintain an E_h in the range in which benzyl viologen (E_0', pH 7, −359 mv) was reduced before isolation of methane bacteria could be achieved. In nutritional studies of *Bacterodies succinogenes* involving dilute inocula, Bryant and Robinson (1958, *unpublished data*) found that erratic results, involving growth or no growth or great variation in the time necessary to reach maximal growth of the organism on a chemically defined medium, could be overcome by using more extreme anaerobic precautions, including the use of indigo carmine indicator (E_0', pH 7, −123 mv) in place of resazurin (E_0', pH 7, about −30 to −40 mv). When the indigo carmine (0.0005 per cent) was colorless, good and reproducible growth was obtained.

In some of the earlier work on the isolation of rumen anaerobes, the media contained no added reducing agent (41, 71). The peptones, extracts, or rumen fluid in the media contained enough reducing materials so that, when very good technique to avoid oxygen was used, good growth was often obtained. However, in most studies various reducing agents have been added. The most commonly used reducing agent is cysteine-HCl but thioglycolate (34, 72, 119), sodium sulfide (72, 81), ascorbic acid (45), and dithionite (120) have been used. Doetsch *et al.* (28) used a combination of cysteine and thioglycolate. There is little good information available on the effect of various concentrations and kinds of reducing agents on bacterial counts from rumen contents. Wilson (130) and Elsden (1956, *personal communication*) obtained about the same counts with cysteine or sulfide but believed that cysteine gave more consistent results. In the isolation of *Methanobacterium ruminantium*, in which case an unusually low E_h was required, Smith and Hungate (120) used low levels of dithionite in early work. However, results were erratic because the poising capacity was low. Higher concentrations of dithionite were toxic. Later in their work, tubes of medium were preincubated with *Escherichia coli* and pyruvate present. The medium was then pasteurized to kill the bacterium and inoculated with little if any exposure to oxygen by injection through the rubber stopper with a needle and syringe. This technique allowed the regular growth of the methanogenic bacteria from high dilutions of rumen contents. It would be of particular interest to compare cultural counts obtained with modifications of this very efficient reducing system to those obtained with the commonly used reducing agents in a less selective medium.

Some work has been done on the effect of various reducing agents on pure cultures of rumen bacteria. No difference in visible growth or final pH was observed in many strains when cysteine-HCl was varied from 0.02 to 0.1 per cent in a rumen fluid-glucose medium (12). Gill and King (45) found cysteine or ascorbic acid to be superior to sulfide or thioglycolate for growth of a strain of *Butyrivibrio*. Allison *et al.* (1) found sodium sulfide to be superior to cysteine for a strain of *Ruminococcus*. Whether this is true for other members of the genus is not known. In nutritional studies on *Bacteroides succinogenes* (Bryant and Robinson (1958, *unpublished data*) and (23)), various lots and concentrations of thioglycolate or ascorbic acid sterilized by filtration, by autoclaving separately, or by autoclaving with other ingredients of a defined medium were unsatisfactory as compared to cysteine or glutathione, which were about equally suitable. Ascorbic acid was sometimes inhibitory in the presence of cysteine. $Na_2S \cdot 9H_2O$ (0.025 per cent) was comparable to cysteine (0.05 to 0.10 per cent) but levels of 0.05 per cent and above tended to inhibit growth. In attempts to determine sulfur requirements, various modifications of the palladium black- or palladium chloride-hydrogen reducing system of Mylroie and Hungate (102) were tried. A gas mixture of 20 per cent hydrogen in carbon dioxide and various concentrations of palladium were used. This reducing system allowed good growth in a rumen-fluid medium but the lowest level of palladium chloride (0.002 per cent) that gave good reduction was toxic in the defined medium.

Although all of the media, developed for nonselectively growing the predominant bacteria of the rumen, are undoubtedly more or less selective, it is evident that some are much more selective than others. The predominant bacteria cultured by some workers from animals on widely varying rations included mainly lactic acid bacteria (5, 109, 110, 129). However, direct microscopic observations in general have indicated that under most conditions gram-negative bacteria are usu-

ally more numerous and many different morphological groups of both gram-positive and gram-negative bacteria occur in large numbers. Nonselective media used by other workers have tended to substantiate this conclusion (13, 34, 92).

It may be that future studies will show a similar occurrence with rumen bacteria to that observed with soil bacteria, *i.e.*, that soil-extract agar without added ingredients was more satisfactory for obtaining isolates representative of the indigenous soil microflora than synthetic media or soil-extract agar enriched with various sources of energy, nitrogen, and other growth factors (86).

Little has been done to evolve truly selective media for specific ruminal bacteria on the basis of pH, temperature range, tolerance of bactericidal or bacteriostatic substances, or nutritional requirements. Bacteria have been selectively isolated for the most part by the use of selective substrates but the media invariably contain at least small amounts of substrates that allow considerable growth of other bacteria.

Selective methods for isolation of cellulolytic bacteria have been reviewed (72, 118), so that only a few more recent observations need be recorded here. These bacteria are usually selectively isolated by use of the zone of cellulose digestion that develops around colonies in cellulose-agar media. Carboxymethyl-cellulose (CMC), a soluble cellulose derivative, has been suggested as substrate for isolation of cellulolytic anaerobes (83, 123). However, only 2 of 32 isolates, about 6 per cent, actively fermented cellulose when this substrate was used. Others have found from 5 to 28 per cent of total isolates from animals on a diversity of rations to be actively cellulolytic even though a nonselective medium was used, containing glucose and cellobiose as substrates (13). It seems possible that the rumen contains species that do not attack comparatively unaltered native cellulose but do attack carboxymethyl-cellulose (53) as has been shown with many nonruminal microorganisms (114).

Anaerobic spirochetes of the genus *Borrelia* have been selectively isolated by means of a technique that took advantage of their ability to migrate through agar and concentrate around zones of hydrolysis formed by cellulolytic bacteria in cellulose agar (10).

Amylolytic anaerobic bacteria are commonly found in very large numbers in the rumen, even when animals are receiving very little starch in the ration. For example, using a nonselective medium, 18, 41, and 56 per cent of the bacterial strains isolated from animals fed rations as divergent as wheat straw, alfalfa hay, and grain mixture, respectively, degraded soluble starch to the point where no iodine-staining material was visible (13). It is evident that selective methods are not necessary for the isolation of many starch-digesting anaerobes and most of these have been isolated using nonselective media or selective media intended for other purposes (5, 11, 12, 17, 19, 40, 46, 50, 60, 68, 69, 72, 74, 75, 130). Hamlin and Hungate (54) enumerated and isolated amylolytic rumen anaerobes directly with starch medium. These workers employed a feed extract-soluble starch medium with the anaerobic technique of Hungate (72) and detected starch digestion by flooding the roll tubes with iodine.

Johns (81) obtained colony counts and isolations of lactate-fermenting bacteria from the rumen using yeast extract-lactate-mineral agar deep tubes with sodium sulfide reducing agent. A control medium without lactate was included. Gutierrez (48) used a similar method but incorporated the anaerobic method of Hungate and peptone in addition to yeast extract. In one of the few cases where bacteria that appear to be of importance in the rumen have been isolated via enrichment techniques involving large inocula, Elsden *et al.* (34) found that *Peptostreptococcus elsdenii* (50) could be enriched by culturing a large inoculum in a yeast extract-starch medium. This culture was then inoculated into the same medium with starch replaced by lactate. The organism was then isolated using agar shake tubes of the lactate medium. If rumen contents were diluted directly into the lactate agar, only *Veillonella alcalescens* (*V. gazogenes*) were obtained. Others (20, 50, 64) have more recently isolated lactate-fermenters utilizing lactate media similar to those of the former workers. Many lactate fermenters have also been isolated from the rumen using nonselective methods (11, 50, 64, 69) indicating that they may be among the predominant rumen bacteria under some conditions.

Gutierrez *et al.* (49) have recently enumerated and isolated saponin-fermenting bacteria, utilizing a rumen-fluid agar and the Hungate anaerobic method. Saponin fermenters were detected by differences in colony size between a control medium and the saponin medium.

Appleby (2) utilized the Hungate anaerobic technique and an agar medium composed of minerals, a carbonic acid (100 per cent CO_2)-

bicarbonate buffer system, and casein to enumerate and isolate proteolytic bacteria. Yeast extract, clarified rumen fluid, or peptone were included. Proteolytic colonies were sometimes detected by zones of clearing around the colonies in roll tubes. However, this was not always satisfactory so that colonies were routinely subcultured into gelatin medium and litmus milk to detect gelatin liquefaction and casein digestion. Rather low total counts of the order of 10^5 to 10^7 per ml of rumen fluid were obtained. The proteolytic bacteria isolated were mainly facultative anaerobes commonly found in sources other than the rumen. Proteolytic counts were about 1 per cent of the total counts.

Fulghum *et al.* (38) used similar methods but found skim milk to produce a more uniformly opaque medium than casein for the detection of clear zones in the roll tube. Proteolytic counts in the rumen-fluid medium averaged 230 million per ml or about 24 per cent of the total count. This percentage is remarkably similar to that obtained by Bryant and Burkey (13) for gelatin-liquefying bacteria isolated using rumen fluid-glucose-cellobiose agar. In this case gelatin-liquefying bacteria accounted for 19 to 28 per cent of total bacteria isolated from animals on a variety of rations. In the latter case all of the gelatin liquefiers were nonsporeforming anaerobes.

The first successful isolation of ruminal methanogenic bacteria that have been shown to be of numerical significance in the rumen has recently been achieved by Smith and Hungate (120). The medium used contained minerals, rumen fluid, and 80 per cent hydrogen-20 per cent carbon dioxide gas as substrate. The success of the medium depended on the establishment of a very low E_h by preincubation of the medium in the presence of pyruvate and *Escherichia coli* as indicated previously. The strain isolated was obtained by first culturing a high dilution of rumen contents in liquid medium and then purifying by culturing in agar roll tubes of the same medium and picking isolated colonies. Counts were also made by culturing high dilutions of rumen contents directly in agar roll tubes and counting the colonies that showed a continuous increase in size over an extended period of incubation.

Gibbons (43) recently isolated a urea-hydrolyzing anaerobic bacterium from rumen contents. Similar bacteria were regularly cultured from relatively low dilutions of rumen contents (10^4 or 10^5 dilutions). The Hungate anaerobic technique with a 1 per cent rumen fluid-glucose-urea-agar roll tube medium with carbon dioxide gas was used. A simple mixture of minerals, methionine, and B-vitamins could be used in place of rumen fluid.

Little work has been done on the possibility of using in rumen studies selective media developed for isolation or enumeration of nonsporeforming anaerobes from other environments. Jensen *et al.* (80) found the selective agar medium for lactobacilli developed by Rogosa *et al.* (117) to be unsatisfactory for enumeration of rumen lactobacilli. If agar was omitted this medium was satisfactory. However, even though incubation was under anaerobic conditions no strictly anaerobic lactobacilli were isolated.

Macdonald and Madlener (89) recently perfected an agar medium for the isolation of *Selenomonas sputigena* (*Spirillum sputigenum*) from the human mouth. The method involves the use of spreading surface growth on veal heart infusion agar plates incubated under hydrogen gas with sheep's blood serum added to rapidly lower the E_h on the agar surface and sodium lauryl sulfate added to selectively inhibit part of the flora. The oral *Selenomonas* species appears to be very closely related to rumen strains (Macdonald, *personal communication*).

Rogosa (116) perfected a trypticase-yeast extract-lactate agar for the selective isolation and enumeration of veillonellae from the oral cavity. The medium contains Tween 80, basic fuchsin, and streptomycin and lactate as the selective agents. No attempt has been made to utilize this medium for the isolation of veillonellae from the rumen. The use of other selective media such as that for fusobacteria (104) and bacteroides (35) has not been attempted for isolation of rumen anaerobes.

D. Maintenance of Cultures

The effort and time involved in maintaining large numbers of cultures of rumen anaerobes can become excessive, for it has been repeatedly observed that cultures stored at refrigerator or room temperature must be transferred at frequent intervals to maintain viability. Solid or semisolid agar shake tubes have usually been used, with transfers every 1 to 3 weeks (5, 28, 34, 46, 119). More frequent subcultures have been necessary with some species (54).

An anaerobic lactobacillus (96) and a butyri-

vibrio (45) have been successfully lyophilized. No other published reports on lyophilized cultures of rumen anaerobes exist. However, on the basis of lyophilized cultures received by the author's laboratory from Gall and co-workers, it is evident that groups isolated by these workers (40, 68, 69) remained viable for at least 1 to 2 years. Also, unpublished data indicate that many of the species cultured by Bryant and co-workers (11, 17–21) have remained viable for at least 1 year when lyophilized in double strength skim milk. However, none of 5 strains of *Bacteroides succinogenes* (15) were viable shortly after lyophilizing.

All species described by the latter workers have been maintained by storage of stab cultures in rumen fluid-glucose-cellobiose agar slants (12) in a dry-ice box with transfers being made at about 6-month intervals. This procedure has been highly successful even with new strains transferred only once after isolation before storage (20). A strain of *Ruminococcus albus* was viable after storage at −10 C for 1 year (36). A strain of butyrivibrio frozen in glycerol according to the method of Howard (66) was viable for at least one year (45).

III. BACTERIAL SPECIES

A. Facultative Anaerobes

Many species of facultatively anaerobic bacteria have been isolated from the rumen. However, in many cases little evidence has been presented to indicate that these bacteria were of numerical importance. These organisms include members of the genera *Flavobacterium* (2, 77), *Pseudomonas* (65), *Proteus* (2, 90), and *Micrococcus* (2, 94, 95).

1. Coliform bacilli. Coliforms do not appear to be of much significance in the rumen of animals so far studied. Counts usually are in the range of 10^3 to 10^6 per ml of rumen contents in sheep (59, 94) and cattle (20, 87, 90), although somewhat higher counts were obtained in some calves at 1 to 3 weeks of age (20). Hobson and Mann (62) could demonstrate very few coliforms in sheep rumen contents by the use of fluorescent antibodies. Heald (58) suggested that coliforms might be of importance in xylose fermentation in the rumen because they were isolated from rumen fluid incubated in the presence of xylose and they rapidly fermented xylose. However, no estimate of numbers was made. The most numerous species of coliform found in the rumen appears to be *Escherichia coli* (90, 94) although *Aerobacter aerogenes* (90), *Aerobacter cloacae* (94), and intermediate types (58) have been isolated.

2. Bacilli. Facultatively anaerobic sporeforming species, *Bacillus subtilis* (65) *Bacillus licheniformis*, *Bacillus cereus*, and *Bacillus circulans* (2) have been isolated but they were found in only small numbers. Appleby (2) cultured similar numbers of bacilli from hay and the rumen but spore counts in the rumen were very low, suggesting that the spores taken in with the hay had germinated. It was suggested that these organisms might be of significance in protein digestion.

3. Propionibacteria. Elsden (30) isolated lactate-fermenting strains of *Propionibacterium* from rumen contents of sheep and cattle using lactate enrichment cultures. Gutierrez (48) later isolated large numbers of a proteolytic, lactate-fermenting, propionic acid-producing bacterium from the rumen of cattle and also from hay and soil. These organisms were identical with *Corynebacterium acnes* (7) except that lactate was fermented. Douglas and Gunter (29) recommended that this species be placed in the genus *Propionibacterium* and later stated that some strains of human origin fermented lactate (48). Organism RO-Cl of Huhtanen and Gall (69) appears to be identical with *P. acnes*. Gutierrez believed this organism to be functional as a lactate-fermenter in the rumen. Bryant *et al.* (20) utilizing the methods of Gutierrez, were unable to detect lactate-fermenting propionibacteria in calves or mature cattle under the conditions studied, though several other lactate-fermenting anaerobes were isolated.

Gyllenberg and Lampila (51) isolated a lactate-fermenting corynebacterium in numbers up to 10 million per ml from the rumen of cattle. They suggested that its presence was accidental because it occurred in large numbers in hay and soil. Complete characteristics were not given but the organism differed from that of Gutierrez in that litmus milk was not peptonized.

4. Lactobacilli. Lactobacilli capable of growth under aerobic conditions have been isolated by many workers. Hungate *et al.* (75) observed that organisms similar to *Lactobacillus brevis* were quite numerous (150 million per ml) in the rumen 22 hr after 3 lb of glucose were given to a sheep on a ration of alfalfa hay. The organism could not be detected among the predominant bacteria cultured at earlier times. One strain of a hetero-

fermentative arginine-decarboxylating lactobacillus was isolated from a sheep fed wheat by Rodwell (115). Briggs (9) isolated a heterofermentative lactobacillus corresponding to her group VII from the rumen of cattle. In neither case was an estimate made of numbers present. Mann *et al.* (93) isolated lactobacilli from the rumen of calves and later Mann and Oxford (96) identified *L. brevis* found in 1:3,000,000 and 1:10,000 dilutions of rumen contents from calves 54 and 32 days of age. A motile homofermentative lactobacillus producing a mixture of inactive and dextrorotatory lactic acids was found in a 1:500,000 dilution from a calf 33 days of age. An anaerobic lactobacillus isolated in this study is discussed below. Mann and Oxford (95) later isolated *Lactobacillus fermenti* from an 11-day-old calf. Lodge *et al.* (87) obtained counts of lactobacilli averaging about 2.5 million per gram from cattle on normal rations for milk production. Jensen *et al.* (80) isolated *Lactobacillus brevis*, *L. buchneri*, *L. fermenti*, *L. plantarum*, *L. acidophilus*, and *L. casei* from a cow receiving a high-roughage ration. The latter two species were found in only low dilutions (10^2 to 10^4) but the others were found in dilutions as high as 10^6 and 10^7. Perry and Briggs (109) found *L. fermenti*, *L. acidophilus*, *L. casei*, and *L. plantarum* in the rumen of calves and mature cattle. *L. fermenti* (to 10^9 dilution) was the predominant lactobacillus in calves fed hay concentrate. *L. acidophilus* (to 10^{13} dilution) was predominant in cows fed hay concentrate and *L. casei* (to 10^{10} dilution) was predominant in cows fed silage.

The studies indicate that lactobacilli capable of aerobic growth may be of functional importance in the rumen under some conditions. The work of Perry and Briggs (109) indicates that they are present in the rumen in such numbers that they would be among the most important species even in animals receiving mainly roughage in the diet. However, the method used by these workers (129) for enumerating the bacteria was quite crude and the fact that either lactobacilli or *Streptococcus bovis* (110) invariably predominated under the cultural conditions used seems to be at odds with direct microscopic observations and pure culture results of most other workers. Hobson and Mann, using the fluorescent antibody technique (62), could not demonstrate *L. brevis*, *L. fermenti*, or *L. acidophilus* in rumen contents of calves or sheep fed a "normal" adult diet. However, a *Lactobacillus* sp. was demonstrated in calves 7 and 24 days of age receiving a milk diet.

Ford *et al.* (37) found the vitamin requirements of rumen strains of *L. casei*, *L. plantarum*, and *L. acidophilus* to be broadly similar to those reported for nonrumen strains. Most of the rumen strains of *L. fermenti* required riboflavin and vitamin B_6 in addition to vitamins reported to be typical of nonrumen strains.

5. Streptococci. Probably the most completely studied and commonly isolated group of bacteria from rumen contents are the facultatively anaerobic amylolytic streptococci identical with or similar to *Streptococcus bovis*. Much of the literature on these bacteria has been reviewed by Briggs (8). In more recent publications, Hungate (74) compared the characteristics of 27 strains isolated from cattle and sheep and determined the numbers in the rumen of cattle fed timothy hay and small amounts of concentrates. Counts ranged from about 0.2 to 140 million per ml with most counts between 1 and 20 million. Gutierrez *et al.* (50) showed that facultatively anaerobic, amylolytic streptococci increased greatly in numbers in the rumen as cattle started to bloat when fed a high-grain, feed-lot, bloat-producing ration. Strains of these bacteria were similar to *S. bovis* except that they lacked its high temperature tolerance, were strongly gram-positive only in 4- to 6-hr cultures, and reduced nitrate. It was shown that these cultures as well as four strains of *S. bovis* had the ability to attach to starch grains. Other strains of *S. bovis* did not appear to have this property (74).

Dain *et al.* (24) confirmed that the slime produced from sucrose by *S. bovis* obtained from many sources was a glucose-containing polysaccharide and showed that CO_2 was required for its production when the organisms were grown aerobically. Tween 80 could substitute for CO_2 when the organisms were grown anaerobically. Using slime production, mannitol fermentation, and reaction in horse blood agar as criteria, 3 varieties of *S. bovis* were detected. The glucose-containing polysaccharide was shown to be a dextran by Bailey and Oxford (3). They confirmed that CO_2 was required for dextran production and that it could be produced in liquid culture, and showed that its production was accompanied by accumulation of fructose and a reducing disaccharide containing fructose.

Ford *et al.* (37) found that none of 26 strains of *S. bovis* required any B-vitamins when grown

under anaerobic conditions, although thiamin stimulated the growth of 5 strains. When grown under aerobic conditions, some strains appeared to require nicotinic acid, biotin, and/or thiamin.

Higginbottom and Wheater (60) confirmed the results of Van der Wath (124), indicating that some amylolytic streptococci present in the rumen produce intracellular iodine-staining material from starch, and identified the strains as *S. bovis*. Hobson and Mann (63) studied factors affecting the production of this polysaccharide, and they showed that fluorescent antibodies against the type specific antigen in the capsule could be used to identify *S. bovis in situ* (62).

S. bovis is undoubtedly involved in the fermentation of starch and other more soluble carbohydrates in ruminant rations. However, on the basis of numbers of organisms found, it seems probable that under most conditions other groups of bacteria, particularly various nonsporeforming anaerobes, are of more significance. The work of Hungate *et al.* (75) indicates that *S. bovis* was intimately involved in the initiation of the very acid ruminal conditions found in acute indigestion caused by rapid ingestion of grain when sheep were maintained on a hay ration. Also, *S. bovis* may be involved in the etiology of feed-lot bloat, as the work of Gutierrez *et al.* (50) suggests. The possibility exists that slime production by *S. bovis* is involved in the stable foam production that is believed to be one of the main causes of bloat due to legumes. However, Bryant *et al.* (see Marston, 97) found only small numbers of streptococci capable of growth under aerobic conditions in cattle bloating on ladino clover.

Facultatively anaerobic streptococci other than amylolytic organisms similar or identical to *S. bovis* do not appear to be of much significance in the rumen. However, small numbers of *Streptococcus liquefaciens* (37), *Streptococcus faecalis*, and unclassified streptococci have been isolated (37, 94, 95) and two thirds of the streptococci isolated from calves approximately 5 weeks old were found to be nonamylolytic (95). Bryant and Burkey (12, 13) found that 11 of 155 strains from a cow fed concentrate were facultatively anaerobic streptococci. One strain was *S. bovis*. The others were long chained, nonhemolytic, nonamylolytic streptococci.

Few facultatively anaerobic cocci other than streptococci appear to be involved in the ruminal fermentation. Bauman and Foster (5) found organisms similar in characteristics to the genus *Pediococcus* in 10^9 and 10^{10} dilutions of rumen contents of cattle fed high-grain rations. A large, gram-negative sarcina apparently seen by several workers in direct microscopic studies, was isolated in small numbers from the rumen of sheep by Heald *et al.* (59). Mann *et al.* (94) found it to be a catalase-positive facultative anaerobe that fermented glucose only and grew well in peptone water. It was named *Sarcina bakeri*. It can be identified in rumen fluid by means of fluorescent antibodies (62).

B. Anaerobes

1. Sporeforming rods. Early work on sporeforming cellulolytic anaerobes indicated that these bacteria were not of importance in the ruminal fermentation (72, 118). Doetsch and Robinson (27) reported that sporeforming anaerobes were insignificant in the rumen. Lodge *et al.* (87) recently reported relatively high spore counts of anaerobes (1.3 to 1.7 million per gram, dry weight basis) for rumen contents of cows. However, neither counts on the feed ingested nor isolations were made. It seems possible that these bacteria were not growing in the rumen. Bullen *et al.* (22) found that *Clostridium perfringens* was rapidly destroyed when introduced into the rumen of normal sheep. Present evidence, particularly from studies on the predominant rumen bacteria, indicates that sporeforming anaerobes are relatively unimportant in the ruminal fermentation as compared to nonsporeforming anaerobes. However, a few species have been characterized that might be of significance.

Hungate (74) recently isolated *Clostridium lochheadii* from a group of cows. This organism represented a considerable proportion of the cellulolytic bacteria enumerated. However, total counts of cellulolytic bacteria were quite low, averaging only 0.26 to 4.8 million per ml. The organism was also found in a tablet of dried rumen bacteria and in a cow in Mississippi. As this bacterium grows readily on a variety of media but has not been previously encountered by Hungate (72) or any of the other workers who have isolated cellulolytic anaerobes from high dilutions of rumen contents, it seems probable that it is usually greatly outnumbered by nonsporeforming cellulolytic anaerobes. Under certain conditions, such as those studied by Hungate (74), it may be of significance. One strain of another clostridium, *C. longisporum*, was found.

In a direct microscopic study, Masson (98) ob-

served sporeforming rods similar to *Clostridium butyricum* to be predominant in the rumen of sheep fed a ration containing a large proportion of flaked maize. *C. butyricum* was isolated but numbers cultured were not given. In a study of proteolytic bacteria in the rumen of sheep fed high concentrate rations, Appleby (2) isolated *Clostridium sporogenes* but the numbers found leave considerable doubt that the organism functioned in the rumen. Gray (47) isolated organisms similar to *Clostridium kluyveri* from the rumen of sheep in numbers varying from nil to 2 million per ml.

2. Nonsporeforming rods.

a. Lactobacilli. Several workers have isolated anaerobic homofermentative lactobacilli from the rumen but only a few have been well characterized. Organism 123, a strain producing levorotatory lactic acid, was found in numbers of about a billion per ml of rumen contents from a 33-day-old calf by Mann and Oxford (96). They considered it a strictly anaerobic variety of *Lactobacillus lactis*. Bryant *et al.* (20) found the same organism to be among the predominant bacteria of 3 calves at 3 and 6 weeks of age but it was not found in younger or older calves or in mature animals. The latter workers isolated another anaerobic species that produced levorotatory lactic acid, designated as the +R3 group, from 6- to 13-week-old calves. It appears to differ from any previously described species. Unpublished results in the author's laboratory showed that anaerobic lactobacilli were among the predominant bacteria in the rumen of 2 cows fed a ration of fresh alfalfa. A detailed study of the characteristics of 2 strains using the methods of Bryant *et al.* (20) indicated that they were identical with the +R3 group from calves except that lactose was not fermented, the titratable acidity in milk was only 0.24 per cent, and growth did not occur at 22 C.

Other isolations of bacteria that appear to be strictly anaerobic, homofermentative lactobacilli have not been well characterized. Unpublished data of Bryant, Barrentine, Shawner, and Williams on the predominant bacteria cultured from the rumen of six cattle pastured on ladino clover (97) indicate that anaerobic lactobacilli similar to those isolated from cattle fed fresh alfalfa, as noted above, accounted for 2 to 15 per cent of the total isolates. Gall and Huhtanen (40) and Huhtanen and Gall (69) found five different groups of homofermentative lactobacilli among the predominant bacteria cultured mainly from calves or mature ruminants fed rations high in grain. The L2 group of Maki and Foster (92), found among the predominant groups of bacteria in cows fed both roughage and grain rations, appears to be a strictly anaerobic, homofermentative lactobacillus.

Strictly anaerobic organisms similar to *Lactobacillus bifidus* (7) have been isolated by several workers. Gibbons (43) cultured a urease-producing organism regularly from low dilutions of rumen contents and named it *L. bifidus* var. *ureolyticus*. The organism required carbon dioxide for growth and produced mainly lactic, acetic, and formic acids in the fermentation of glucose. Lactic acid accounted for 69 to 85 per cent of the total acids produced. The optical rotation of the lactic acid produced was not determined. Wasserman *et al.* (125, 126) isolated an anaerobic, bifid, inactive lactic acid-producing *Lactobacillus* in large numbers from cattle fed a ration of timothy hay supplemented with urea and molasses. Bauman and Foster (5) isolated organisms similar to *L. bifidus* in large numbers from cattle fed an alfalfa hay ration. These organisms produced acetic and lactic acids in 2:1 ratio. The optical rotation of the lactic acid produced was not determined. Hungate *et al.* (75) showed that a bifid gram-positive rod increased greatly in numbers in a sheep's rumen dosed with 4 lb of cracked corn after being on a ration of alfalfa hay.

Although complete characteristics were not given, the inactive lactic acid-producing organism of Wasserman *et al.* (125, 126) appears to be most closely related to *L. bifidus* type I of Weiss and Rettger (128). Whether the other strains isolated were more closely related to this organism than to the more anaerobic variety, *L. bifidus* type II of Weiss and Rettger (*L. parabifidus*), producing dextrorotatory lactic acid, is not known.

The organism 123, which Mann and Oxford believed to be an anaerobic variety of *L. lactis*, produced a small amount of gas in glucose agar shake tubes (20, 96). However, other characteristics indicate that it is essentially a homofermentative organism. The only other gas-producing anaerobic organism isolated from rumen contents that might be a lactobacillus is the RO-T organism briefly described by Gall and Huhtanen (40).

b. Ramibacteria. Bryant *et al.* (20) isolated two apparently new species of gram-positive, branching rods that appear to belong in the genus *Ramibacterium* Prévot (7). The +R1 group was

found only among the predominant bacteria isolated from 1-week-old calves. It produced a large amount of succinic acid and small amounts of acetic, formic, and lactic acids and no gas in glucose medium. The organism showed some similarities to strains of *L. bifidus* (*L. parabifidus*, Weiss and Rettger (128)) studied by Pine and Howell (111) in that succinic and formic acids were produced and bicarbonate was required for good growth. However, the relatively large amount of succinic and small amount of lactic acid produced by the +R1 organism appears to separate it from *L. bifidus*.

The other species of *Ramibacterium* isolated was a strict anaerobe found also in young calves (20). This organism produced lactic, acetic, and formic acids in glucose medium but was isolated using a lactate agar and was found to ferment lactate as indicated by the production of volatile fatty acids. The individual acids produced in the lactate fermentation were not determined.

c. Eubacteria. In a preliminary study of a large number of strains of predominant bacteria from the rumen of cattle, Bryant and Burkey (12, 13) isolated two groups of anaerobic, small, gram-positive rods. The +SR-gGXC group was subsequently studied in detail (+R2 group (20)) and placed in the genus *Eubacterium* Prévot (7). This organism produced gas, including hydrogen, and acetic, formic, and lactic acids in similar proportions in glucose medium. It rapidly liquefied gelatin. It appeared to differ from many species of *Eubacterium* in that propionic or butyric acids or ammonia were not produced and from others such as *E. biforme* or *E. aerofaciens* (7) in carbohydrates fermented, gelatin liquefaction, and production of a larger amount of volatile acid (108). This species never accounted for more than 3 per cent of the total bacteria isolated from the rumen (13).

The ±CR-GXC group of Bryant and Burkey (12, 13) has since been studied in detail and the results will be presented here. The methods of isolation of strains and of detailed studies have been previously described (12, 17). Twenty strains selected for study were isolated from eight different cows on five different rations. Two animals fed fresh alfalfa yielded four strains; two animals fed alfalfa hay, eleven strains; one animal fed alfalfa hay and a grain mixture, two strains; two animals fed alfalfa hay, corn silage, and grain, one strain; and one animal fed soybean hay and grain, two strains.

All strains were nonmotile small short rods (0.4 to 0.7 μ by 0.7 to 1.5 μ) with many cells almost coccoid. They were arranged mainly singly, in pairs, and in short chains, with occasional longer chains of 20 or more cells. They were weakly gram-positive in 16-hr cultures with many cells not retaining the crystal violet. Older cultures were gram-negative. No definite capsules or iodine-staining substances were observed. Little variation was noted in morphology except that a few strains tended to be more coccoid than the others.

Surface colonies in rumen fluid-glucose-cellobiose agar roll tubes were entire, smooth, slightly convex, translucent to opaque, and light buff in color. They were from 2 to 4 mm in diameter after 3 days of incubation. Deep colonies were lenticular.

Growth in liquid glucose medium was evenly turbid in 16 to 18 hr.

There was considerably more visible growth in rumen fluid-glucose medium than in the same medium minus rumen fluid but with 0.5 per cent of yeast extract and 1.5 per cent of trypticase added.

None of the strains grew at 22 or 50 C. All strains grew well at 30 and 37 C but only 3 strains grew at 45 C. All strains were strict anaerobes.

The final pH in lightly buffered glucose medium was from 5.0 to 5.5.

None of the strains produced catalase, indole, or hydrogen sulfide; reduced nitrate; liquefied gelatin; hydrolyzed cellulose or starch; fermented gum arabic, glycerol, mannitol, inulin, or lactate.

All strains fermented glucose, cellobiose, and fructose.

Other characteristics varied. Eighteen of the 20 strains fermented D-xylose, L-arabinose, and lactose; 11 strains fermented maltose, dextrin and sucrose; 9 strains fermented xylan; 7, salicin; and 5, esculin. Eleven strains gave positive Voges-Proskauer tests.

All strains studied (table 1) produced lactic, formic, acetic, and butyric acids. All strains in table 1 were rechecked for gas production in glucose agar shake tubes (19) and for hydrogen production in glucose liquid medium (21). None of them caused gas splits in the agar nor was hydrogen detected. It seems possible that the hydrogen detected in the original culture of strain B_1C23 was due to contamination, although microscopic examination of the culture and the similarity of the products recovered to those of

TABLE 1

*Some fermentation products of a Eubacterium species from the bovine rumen when grown in rumen fluid-glucose medium**

Product	Strain				
	B4	B1C26	GA195†	B134	B1C23†
	mmoles/100 ml medium				
Hydrogen	0	0	0	0	0.20
Carbon dioxide			0.33		0.15
Lactic acid	3.22	2.67	0.71	0.12	0.88
Formic acid	3.52	2.84	0.93	1.96	0.72
Acetic acid	0.60	0.48	0.17	1.32	0.15
Butyric acid	1.30	1.18	0.71	0.41	0.47

* No methane, ethanol, succinic acid, or propionic acid was detected.

† The products of these strains were determined in lowly buffered medium with a nitrogen gaseous phase (17). The others were grown in a highly buffered medium with a carbon dioxide gaseous phase in which carbon dioxide was not determined (19).

the other strains indicated otherwise. None of the 5 strains in table 1 produced ammonia from trypticase, using the method of Bryant *et al.* (19).

Because this group of bacteria includes gram-positive, strictly anaerobic, nonmotile, nonbranching, regularly shaped rods that ferment carbohydrates with the production of lactic, formic, butyric, and acetic acids, it belongs in the genus *Eubacterium* Prévot (7). It differs from all known species of the genus in several characteristics. It differs from many in its failure to produce abundant gas, ammonia, and ethanol and from some in its failure to liquefy gelatin and produce hydrogen sulfide or propionic acid, as well as in many carbohydrates fermented. It has some similarities to *Corynebacterium avidum* (Eggerth) Prévot (7) in that it is a gram-positive anaerobe that produces lactic and butyric acids but differs in not liquefying gelatin, not producing hydrogen sulfide, fermenting arabinose but not glycerol, and in morphology. It is similar to *Butyribacterium rettgeri* (7) in many characteristics but differs in producing formic and lactic acids in addition to acetic and butyric acids, in its inability to ferment lactate, and in the fermentation of several carbohydrates.

The name *Eubacterium ruminantium* n. sp. is proposed for all strains having the characteristics listed above. The type strain is GA195. Thirteen of the 20 strains fit into 2 biotypes on the basis of the variable characteristics. In addition to the characteristics that were invariable for all 20 strains, these 13 strains all fermented D-xylose, L-arabinose, and lactose. Eight of them (biotype 1) were Voges-Proskauer positive; fermented maltose, dextrin, and xylan; and did not ferment sucrose, salicin, or esculin. The reverse was true of 5 strains (biotype 2). None of the other 7 strains could be placed in further biotypes because none of them were identical in characteristics. They seemed to be intermediate between biotypes 1 and 2 except that 2 strains failed to ferment xylose and arabinose and 2 other strains failed to ferment lactose.

Strains of biotype 2 were isolated from only the two animals fed alfalfa hay. Strains of biotype 1 were isolated from animals on all rations listed above, whereas the 7 strains not placed in either biotype represent isolations from animals on all rations except soybean hay and grain.

The results of this study suggest that most if not all of the strains previously placed in the ±CR-GXC group (12–14), belong to the species *E. ruminantium*. The group represented from 3.3 to 7.3 per cent of total isolates in animals fed alfalfa hay, alfalfa silage, fresh alfalfa, alfalfa hay and grain, or blue grass pasture and grain. None were isolated from animals fed wheat straw, fresh ladino clover (97), or grain mixture only. This indicates that *E. ruminantium* is an important ruminal organism in cattle fed many of the common predominantly roughage rations.

Maki and Foster (92) studied an anaerobic, gram-positive rod that produced mainly propionic, acetic, and lactic acids from glucose. The organism was among the predominant bacteria isolated from cattle fed hay. Other characteristics were not determined. This organism might represent a species of the genus *Eubacterium*.

d. Methanobacteria. The production of methane is one of the most characteristic of the reactions known to occur in the rumen. Cultures of *Methanosarcinae* (6), *Methanobacterium formicicum* (105, 131) and *Methanobacterium sohngenii* (105) were probably obtained in pure culture from enrichment cultures inoculated with large volumes of rumen contents. Whether these species are of functional significance in the rumen is in doubt. Recently, Smith and Hungate (120) cultured an anaerobic, gram-positive, nonmotile, methanogenic, short rod from rumen contents of cattle and sheep in different geographical locations. They

demonstrated that it occurred in numbers from 1×10^6 to 2×10^8 per ml. The purity of one strain was very definitely established and it was shown to utilize hydrogen and formic acid but not other substrates in the formation of methane. The organism was named *Methanobacterium ruminantium*.

e. Lachnospirae. Only two species of anaerobic, nonsporeforming, motile, gram-positive rods have been isolated from rumen contents and only one of these has been found regularly. Bryant and Small (18) described the genus *Lachnospira* as anaerobic, nonsporeforming, monotrichous, weakly gram-positive, curved rods that fermented glucose with the production of large amounts of ethanol and lactic, formic, and acetic acids. The single species placed in the genus, *L. multiparus*, produces a very characteristic filamentous colony; some cells are usually arranged in long chains and show little tendency to curve. These characteristics plus the Gram reaction suggest a relationship to the order *Actinomycetales* Buchanan (7). However, the genus was tentatively placed in the tribe *Spirilleae* Kluyver and van Niel of the family *Pseudomonadaceae*, placing emphasis on the characteristic curved to helicoidal cells and monotrichous and polar flagellation. However, no other genera of the tribe were gram-positive. Using the classification of the seventh edition of *Bergey's Manual of Determinative Bacteriology* (7), the genus is provisionally placed in the family *Spirillaceae* Migula, although none of the recognized genera of this family are gram-positive.

Early work indicated that *Lachnospirae* never represented a major portion of the strains of bacteria isolated from rumen contents. They accounted for 1 to 3 per cent of total isolates from animals on a wide variety of rations. However, more recent studies indicated that the genus accounted for 16 to 31 per cent of the total isolations from six animals pastured on bloat-provoking ladino clover (97).

f. Cillobacteria. On the basis of its being an anaerobic, peritrichous, gram-positive rod, a single strain isolated from a 1 to 500 million dilution of rumen fluid from a cow pastured on clover was placed in the genus *Cillobacterium* Prévot (7) and named *C. cellulosolvens* (21). It differed from other species of the genus by failing to produce gas, by producing lactic acid homofermentatively, and by its ability to digest cellulose. The fact that other strains of this bacterium have not been isolated suggests that it is not an important ruminal species.

g. Succinic acid-producing Bacteroides. Strictly anaerobic, nonmotile, gram-negative rods that produce large amounts of succinic acid in the fermentation of carbohydrate appear to be among the more important bacteria present in the rumen. The presence of these and many other succinic acid-producing bacteria (19) suggests that the quantitatively important production of propionic acid in the rumen proceeds to a considerable extent through the action of these bacteria on carbohydrate to produce succinic acid, which is then decarboxylated to propionic acid by other ruminal microorganisms (27). The succinic acid-producing *Bacteroides* are concerned with the digestion of many carbohydrates, including quantitatively important polysaccharides such as cellulose, xylan, and starch and appear to be concerned in protein degradation.

Three ruminal species of this group, *Bacteroides succinogenes* Hungate (7, 15, 72), *B. amylophilus* Hamlin and Hungate (54), and *B. ruminicola* Bryant *et al.* (19), have been named. As data accumulate on additional characteristics and on additional strains of this group of bacteria, the problem of delineating species becomes increasingly difficult.

Although a considerable amount of variation occurs in morphology and pigment production, all cellulolytic strains of the group that have been studied appear to represent one quite well defined species, *B. succinogenes*, on the basis of physiological and cultural characteristics (15, 20, 71, 72). Also, nutritional studies on strains selected on the basis of the differing cell shape and size, pigment production, and small differences in carbohydrates fermented, showed that all strains required certain volatile fatty acids for growth (16) and that B-vitamin and nitrogen requirements were very similar (23).

B. amylophilus of Hamlin and Hungate (54) is well differentiated from the other succinic acid-producing *Bacteroides* in that it ferments only starch and maltose. All of these other bacteria ferment glucose and, usually, many other carbohydrates.

B. ruminicola of Bryant *et al.* (19) includes succinic acid-producing *Bacteroides* that usually ferment a wide variety of carbohydrates, including glucose, starch, and xylan but not cellulose. This species includes strains that vary considerably in characteristics but these characteristics

did not correlate well enough to justify splitting it into more than one species on the basis of present knowledge. The species was, however, divided into two subspecies. *B. ruminicola* subsp. *ruminicola* included eight biotypes that differed in hydrogen sulfide production, gelatin liquefaction, and carbohydrates fermented. *B. ruminicola* subsp. *brevis* included three biotypes that differed in hydrogen sulfide production and the fermentation of a few carbohydrates and differed from the first subspecies by being more coccoid and by growing well in medium in which trypticase and yeast extract were added in place of rumen fluid. Group D of the more numerous carbohydrate-fermenting bacteria isolated from the rumen of sheep by Wilson (130) appears to be identical with *B. ruminicola* subsp. *brevis*.

In work on predominant bacteria in young calves Bryant *et al.* (20) isolated strains from relatively old calves that fit well into the species *B. succinogenes* and *B. ruminicola*. However, from 1- and 3-week-old calves the R1 group was isolated that differed from all previously described succinic acid-producing *Bacteroides* in that they grew at 22 C and produced indole and some propionic acid. This R1 group was split into three subgroups on the basis of ammonia production, gelatin liquefaction, and carbohydrates fermented. Group R1-a appeared to be closely related to *B. amylophilus* because it fermented only starch and its hydrolytic products. However, *B. amylophilus* (54) does not ferment glucose. Groups R1-b and c were more closely related to *B. ruminicola*.

Maki and Foster (92) isolated an anaerobic succinic acid-producing gram-negative rod from the rumen of cattle that might belong to this group of bacteria. However, few characteristics were given.

h. Butyric acid-producing Bacteroides. Bryant *et al.* (20) isolated from the rumen of young calves an anaerobic, lactate-fermenting, gram-negative, nonmotile pleomorphic rod (R2 group) that produced gas, including hydrogen, butyric and acetic acids, and a small amount of propionic acid in glucose medium. The organism was placed in the genus *Bacteroides* (7). It also could be placed in the genus *Sphaerophorus* Prévot (7) but the present author sees no good reason for not considering species of this genus as belonging to the genus *Bacteroides* on the basis of known characteristics of the two groups. The only difference between these groups is that *Sphaerophorus* spp. in general appear to be somewhat more pleomorphic.

It is difficult to compare the characteristics of the R2 group with those of recognized species of the two genera, because in most cases fermentation products have not been determined. However, it differs from all species in several characteristics. It is similar to some species of *Sphaerophorus* in that butyric acid is produced but differs from all of these by failing to produce lactic acid and from the individual species in several characteristics. It is the only organism of these groups known to ferment lactate but this characteristic has not been determined for most species.

Doetsch *et al.* (26) described an anaerobic, gram-negative, nonmotile curved rod that produced butyric and acetic acids and small amounts of propionic and lactic acids in the fermentation of xylose. The organism was provisionally placed in the genus *Bacteroides* and named *B. amylogenes*. The typically curved shape of the cells suggested to the authors a relationship to the genus *Desulfovibrio* but absence of flagella and failure to reduce sulfate argued against this consideration. Most characteristics of the organism suggest a close relationship to the species *Butyrivibrio fibrisolvens* Bryant and Small (17). However, the latter species is motile and possesses monotrichous flagellation and unpublished data indicate that it does not produce iodine-staining intracellular polysaccharide, whereas *Bacteroides amylogenes* does so.

i. Fusobacteria. Huhtanen and Gall (69) quite regularly isolated a group of gram-negative, anaerobic rods with pointed ends from relatively low dilutions of rumen contents. These organisms produced acids other than lactic, gas, and a foul odor in a highly fortified glucose medium. The few characteristics studied indicated that the organisms belonged in the genus *Fusobacterium* Knorr (7) but did not allow specific identification.

Bryant *et al.* (19) isolated an apparently new nonmotile species of the genus *Fusobacterium* from the rumen of 1- and 3-week-old calves but did not find similar organisms in older animals. Strains varied in some physiological characteristics such as indole production, gelatin liquefaction, starch hydrolysis, fermentation of trehalose and mannitol, and growth at 22 and 45 C, but morphological and other physiological characteristics including fermentation products were very similar,

indicating a group of very closely related organisms.

This group was similar to some oral strains of fusobacteria, *Fusobacterium fusiforme* (*F. plauti-vincenti* Knorr) (7), in that lactic acid was an important fermentation product, butyric acid was not detected (78), and the size and shape of cells were similar. However, they differ from this organism in several characteristics, including the production of ammonia, and differ from all oral strains of fusobacteria by producing considerable gas, including hydrogen. They are similar in many characteristics to the gas-producing species *Fusobacterium biacutus* Weinberg and Prévot (7, 112), isolated from the appendix, but differ in having larger and longer cells and in producing lactic and not propionic acid.

j. Butyrivibrio. In studies on the cellulolytic anaerobes of the rumen, Hungate (72) isolated a small, gram-negative, curved rod that fermented a wide variety of carbohydrates and produced carbon dioxide, hydrogen, butyric and formic acids, and showed an uptake of acetic acid in the fermentation of cellulose. Subsequently, Bryant and Small (17) studied the characteristics of a large number of strains of similar organisms isolated in large numbers from cattle fed a variety of rations. The genus *Butyrivibrio* was established with the type species *B. fibrisolvens.* The genus included anaerobic, nonsporeforming, monotrichous, gram-negative, curved rods that fermented glucose with the production of butyric acid. Strains placed in the type species produced fermentation products similar to those of the strain of Hungate, except that a production of acetic acid occurred in some strains. Some individual strains varied considerably in characteristics but most of the variations were small and failed to correlate, so that most strains were placed in the type species and no other species were named. Only 3 of the 48 strains studied could be shown to ferment cellulose but many of the strains hydrolyzed starch and fermented xylan, inulin, salicin, esculin, and many mono- and disaccharides. Wilson's group C, isolated from sheep, fits the species and was shown to ferment grass levan (130). Saponin-fermenting strains isolated by Gutierrez *et al.* (49) resembled an atypical strain studied by Bryant and Small (17) that failed to produce hydrogen. It is evident that members of this genus attack a variety of carbohydrates that are of importance in ruminant nutrition. This genus appears to be of importance in protein degradation, as indicated by casein and gelatin digestion by some strains.

Gill and King (45) working with a strain of *Butyrivibrio*, showed that butyric acid production was greatly lowered and lactic acid production was greatly increased when fermentation products produced from glucose were determined in a synthetic medium as compared with a medium containing rumen fluid. Whether this might be true for other media containing no rumen fluid or for other strains is not known, but it is a point to consider in attempting to identify similar strains of bacteria which might produce little or no butyric acid. In addition, these workers found that carbon dioxide was required for growth in the synthetic medium while the strains of Bryant and Small (17) did not require addition of carbon dioxide to rumen-fluid medium.

In addition to the isolations of the genus *Butyrivibrio* listed above, other workers have isolated strains of ruminal bacteria that appear to belong to this genus, although too few characteristics were determined to be certain. These include the RO-H types described by Huhtanen and Gall (68) and the B-1 group of Maki and Foster (92). So far as is known, members of the genus *Butyrivibrio* have not been isolated from sources other than the rumen.

k. Succinivibrio. The genus *Succinivibrio* Bryant and Small (19) includes anaerobic, nonsporeforming, gram-negative, small curved rods with monotrichous polar flagellation. They produce a large amount of succinic acid in the fermentation of glucose. Bryant and Small studied 7 strains from cattle and placed all of them in 1 species, *S. dextrinosolven.* The strains were very similar in characteristics except for variation in fermentation of a few carbohydrates. One strain lost the characteristic helicoid shape of the cells after it was maintained in the laboratory for some time.

Group B of the carbohydrate-fermenting bacteria isolated from the rumen of sheep by Wilson (130) and Elsden (1956, *personal communication*) belonged to this species. It was identical in carbohydrate fermentation with strain D158 of Bryant and Small except that starch was fermented. The three strains of Wilson did not show the helicoid shape characteristic of the strains of Bryant and Small. Also, when grown in a medium devoid of sodium bicarbonate or in a medium with a low level of yeast autolysate, these strains produced large, swollen, unevenly staining forms 3 to 5 μ by 12 to 50 μ.

This species produces mainly succinic and acetic acids in the fermentation of glucose; it may produce some formic and lactic acids and show an uptake of carbon dioxide. It does not appear to be active in protein catabolism but actively ferments several carbohydrates. The fact that these organisms were found in greater numbers in animals fed rations high in grain, plus the fact that maltose and dextrin were actively fermented, indicated that the species may be of importance in the rumen as a fermenter of the hydrolytic products of starch. Wilson's strains were shown to ferment grass levan.

l. Desulfovibrio. During a study of lactate-fermenting bacteria from the rumen of cattle, Gutierrez (48) isolated small, gram-negative, curved rods that produced black colonies in the presence of ferrous sulfate. In addition to lactate fermentation, strains attacked malate. The organisms were found in relatively small numbers, 50,000 to 100,000 per ml, indicating that they were relatively unimportant under the conditions studied.

m. Selenomonas. Selenomonas ruminantium (Certes, 1889) Wenyon, 1926 (7) was one of the first rumen bacteria named and studied. It was recognized by its crescent shape and by the attachment of a tuft of flagella to the concave side of the cell as well as by its internal structure. Lessel and Breed (84) have reviewed the early literature and taxonomy of this organism. It was not until 1955 that the species was obtained in pure culture and identified as *S. ruminantium* (11). It was shown that the RO-HD types cultured earlier by Huhtanen and Gall (68) also belonged to this species.

The 10 strains of *Selenomonas* studied were shown to be strict anaerobes that ferment a wide variety of carbohydrates and produce an unusually low pH of 4.3 to 4.5 in glucose medium as compared to other rumen anaerobes with the exception of the lactic acid bacteria. In the fermentation of glucose, lactic, acetic, and propionic acids accounted for most of the products. Strains varied from those that produced mainly lactic acid to those that produced mainly propionic and acetic acids. Unpublished data on two strains indicate that the lactic acid is optically inactive. Carbon dioxide was detected quantitatively but strains do not produce gas splits in glucose agar shake tubes. Three strains fermented lactic acid with the production of propionic and acetic acids, suggesting a similarity to propionibacteria. Although some variation occurred between strains in morphology, cultural characteristics, and the fermentation of a few carbohydrates, the differences did not appear great enough to warrant establishing more than one species. As lactate fermentation may be of considerable importance in the rumen, the lactate-fermenting strains were given variety status and named *S. ruminantium* var. *lactilytica.*

Three species of the genus are recognized, primarily on the basis of habitat, and it has been suggested that when more complete data are available it may be found that all organisms belong to the same species (84). *Selenomonas palpitans*, the species observed in the caecum and intestines of rodents and herbivores, has not been studied in pure culture. *Selenomonas sputigena* (*Spirillum sputigenum*), the species of the human oral cavity, has been isolated and studied by many investigators. Macdonald (88) has reviewed the literature on this organism and studied the characteristics of additional strains. Although fermentation products have not been determined and the fermentation of few carbohydrates is recorded, this species appears to be identical with *S. ruminantium* except in the ability of the latter organism to produce hydrogen sulfide (11). Macdonald (*personal communication*) has shown that oral strains failed to produce hydrogen sulfide even when grown on medium prepared in the author's laboratory, whereas rumen strains vigorously produced it. However, a strain of *Selenomonas* that failed to produce hydrogen sulfide was recently isolated from the rumen of a calf (20). The information available suggests that *S. ruminantium* and *S. sputigena* are not distinct enough to be considered separate species. It seems best, however, to consider strains from the different habitats as separate species until the type species, *S. palpitans*, has been isolated and its characteristics compared with the other species.

n. Other motile, curved rods. During study of strains of organisms similar in shape to selenomonads (11), strain B385, isolated from a young calf, was found to differ significantly in physiological characteristics from *Selenomonas ruminantium*. It failed to produce hydrogen sulfide; had a higher final pH of growth; did not ferment salicin or esculin; did ferment xylan; and produced butyric, lactic, and formic acids and utilized acetate in the glucose fermentation. The cells had a tendency to be less curved than *Selenomonas* strains and fla-

gella occurred in tufts that appeared to be attached at random over the surface of cells rather than primarily to the concave side of the cell. This organism was similar to *Butyrivibrio fibrisolvens* (17) except that the latter organism is smaller, monotrichous, and usually ferments esculin and salicin.

On the basis of shape and type of flagellation, strain B385 belongs in the family *Spirillaceae* Migula (7) and fits the genus *Spirillum* except that this genus does not include strict anaerobes. It appears to represent a new genus of bacterium.

Similar organisms have recently been found among the predominant bacteria in the rumen of cattle fed on ladino clover pasture (Bryant and Robinson, 1958, *unpublished data*, and (97)). Six strains studied were identical and similar to strain B385 except that they fermented esculin and salicin and failed to ferment xylose and xylan; also, the tufts of flagella were usually attached at the ends of the cell. Fermentation products have not yet been determined.

o. Borrelia. Small helicoid organisms can almost always be observed in direct microscopic preparations of rumen contents. In the isolation of cellulolytic bacteria (71, 118), considerable difficulty has been encountered in freeing them from such small helicoid organisms. Bryant (10) isolated several strains from the rumen of cattle and sheep and studied cultural and physiological characteristics of one strain. This organism was a small, irregularly coiled spirochete that stained with aniline dyes. This property placed it in the genus *Borrelia*. The organism was a strict anaerobe that required unknown substances present in rumen fluid for growth and was greatly stimulated by inclusion of fermentable carbohydrates, many of which were utilized. Glucose was fermented with the production of large amounts of succinic and acetic acids and small amounts of lactic acid, formic acid, ethanol, and carbon dioxide.

On the basis of morphology and colony type, most spirochetes isolated (10, 12) appeared to be similar to the *Borrelia* spp. studied in detail but one strain isolated from a cow and one from a sheep were larger and more loosely coiled. Further work would be necessary to determine if more than one species is present in the rumen. A lack of knowledge on the physiology of spirochetes makes it difficult to determine the relationship of the rumen borreliae to those from other sources. However, in morphology and in the requirement of anaerobic conditions for growth, the rumen borreliae are similar to such oral spirochetes as *Borrelia vincentii*.

p. Succinimonas. A new genus and species of anaerobic, starch-hydrolyzing, succinic acid-producing, gram-negative, monotrichous, oval rod, *Succinimonas amylolytica*, was recently described (19). It has been regularly isolated from the rumen of cattle fed rations containing grain mixtures, though always outnumbered by other starch-hydrolyzing bacteria.

Jamieson and Loftus (79) described the occurrence and characteristics of a filamentous organism of the family *Beggiatoaceae*, which they observed in rumen contents of sheep fed rations that lead to low ruminal ammonia levels. This organism was not obtained in pure culture.

3. Cocci.

a. Peptostreptococci. Numerous, apparently different species of anaerobic cocci have been isolated from rumen contents, although many of them have not been adequately described. Most of these bacteria can be placed in the genus *Peptostreptococcus* Kluyver and van Niel as modified by Smith (7) on the basis of their being gram-positive and occurring in pairs or chains. The current status of classification of anaerobic cocci is poor because of many inadequate descriptions of species and the recognized influence of medium ingredients, such as oleic acid and thioglycolate, on morphological and physiological characteristics used in their classification (7). Hare and associates (55, 122) were able to divide many strains of anaerobic cocci from human sources into only nine different groups. It seems probable that future comparative studies of physiological and morphological characteristics of species of the genera, *Veillonella*, *Peptococcus*, and *Peptostreptococcus* now included in *Bergey's Manual of Determinative Bacteriology* (7) will show that some are identical and that, on the basis of physiological characteristics, new genera or subgenera will be established.

Several groups of ruminal cocci that belong in the genus *Peptostreptococcus* and appear to be homofermentative lactic-acid bacteria have been described. The RO-SCC and RO-LCC groups of Huhtanen and Gall (69) and the L1 group of Maki and Foster (92) cannot be compared with recognized species because they were not described in detail. The C3 group of Bryant *et al.* (20), isolated from young calves, appeared to be most closely related to the non-gas-forming species of human origin *P. intermedius* (7), and group

VIa of Hare and associates (55, 122). It differs from these organisms in several characteristics including its tendency to form pointed ends. *P. intermedius* produced propionate in addition to lactate and formate whereas the C3 organism produced mainly lactate with small amounts of acetate and formate.

Maki and Foster (92) isolated and briefly described anaerobic, gram-positive cocci that form long chains and produce mainly propionic and lactic acid in the glucose fermentation. These characteristics suggest a relationship to *P. intermedius* and similar species.

The C1 group (20) of large, chain-forming, lancet-shaped cocci isolated from the rumen of 1-week-old calves was placed in the genus *Peptostreptococcus* but could not be identified with known species. It was similar to some species in producing gas and ammonia but different because it did not produce combustible gas, grew over a wider range of temperatures, and fermented a wider variety of carbohydrates. Acidic end products, mainly acetate, lactate, and succinate, also differed from those of recognized species. The C1 group appeared to be identical in the characteristic morphology and list of carbohydrates fermented with the anaerobic streptococci isolated from turkey feces by Harrison and Hansen (56). Other characteristics of the latter organisms were not determined.

Elsden *et al.* (31) and Elsden and Lewis (32) isolated from the rumen of sheep an anaerobic, gram-negative coccus that fermented lactate and several carbohydrates with the production of volatile fatty acids including valeric and caproic acids. The formation of fatty acids and the fermentation of certain amino acids and acrylic acid (85) by this LC coccus were studied in detail. Huhtanen and Gall (69) regularly found similar organisms, the RO-C8 group, that produced butyric and longer chained volatile fatty acids, from the rumen of calves and adult ruminants fed high-grain rations, but detailed studies were not made. This organism was described as being gram-positive. Elsden *et al.* (34) later isolated more strains of the LC coccus and performed a detailed systematic study of their characteristics. Naming of this organism was deferred because it did not fit the description of any known genus. The authors found that it had some similarities to *Neisseria* and *Moraxella*. More recently, organisms similar to the LC coccus have been isolated in large numbers from calves (20, 64) and from mature cattle on a feed-lot, bloat-provoking ration (50). The organisms from calves (20) were identical with the LC coccus except that sucrose was fermented and some strains fermented glycerol and grew at 45 C. The isolates from cattle failed to ferment sorbitol, were variable in sucrose and glycerol fermentation, and grew at 45 C. Hobson *et al.* (64) found 3 distinct serological groups in 211 strains from calves. Gutierrez *et al.* (50) considered their strains identical with the LC coccus. Although the strains were predominantly gram-negative, some strains showed cells which had a tendency toward being gram-positive, and some cells showed gram-positive granules. The organism was placed in the genus *Peptostreptococcus* because its morphology and fermentation products were similar to species now placed in this genus. It was named *P. elsdenii*. It has been suggested that this organism might be found to be identical with *P. lanceolatus* (Prévot) Smith (7) if studied under comparable conditions (20).

b. Ruminococci. Among the first groups of bacteria shown to be of importance in cellulose digestion in the rumen were the anaerobic cellulolytic cocci. The early work with these organisms and other ruminal cellulolytic bacteria has been reviewed by Sijpesteijn (118) and Hungate (72). Since these workers first isolated strains of these bacteria, many workers have isolated them from high dilutions of rumen contents of cattle and sheep (12, 13, 20, 21, 74, 82, 83, 91) and from caecal contents of rabbits (52). Sijpesteijn (118, 119) established the genus *Ruminococcus* to include gram-positive, nonmotile, nonsporeforming, anaerobic cocci that fermented cellulose with the production of large amounts of succinic acid. The three strains studied were placed in the type species *R. flavefaciens*. Neither the species nor the genus could include strains described by Hungate (72), because these strains produced little or no succinic acid and some were gram-negative. On the basis of a study of more strains of cellulolytic cocci isolated from both cattle and sheep, Hungate (74) broadened the description of the genus to include gram-negative or variable cocci that ferment carbohydrate to form acetate, at least traces of hydrogen, and various combinations of ethanol, formate, lactate, and succinate. Fourteen strains isolated from cattle by Bryant *et al.* (21) fit the description of the genus *Ruminococcus* Sijpesteijn as modified by Hungate, except that production of neither hydrogen nor other gases

could be detected in eight of the strains. It was believed that these should be included in the genus.

Sijpesteijn (118), following the taxonomy of Kluyver and van Niel, indicated that the cellulolytic cocci should be included in the tribe *Streptococceae* but recognized them as a new genus because they were not true lactic-acid bacteria that could be included in the genera *Streptococcus* or *Betacoccus* and because they were not protein-fermenters that could be included in the genus *Peptostreptococcus*. In the seventh edition of *Bergey's Manual of Determinative Bacteriology* (7), Smith has broadened the latter genus to include all gram-positive anaerobic cocci that occur in pairs or chains. This would include the species of *Ruminococcus*. However, the genus *Peptostreptococcus* (Kluyver and van Niel) Smith includes species of such diverse physiological characteristics that it seems probable that in the future some species will be placed in different genera or subgenera. For the present, it seems best to retain the genus *Ruminococcus*.

The species *R. flavefaciens* Sijpesteijn (119) included the ruminococci that produced succinic acid and yellow pigment on cellulose, fermented cellulose and cellobiose, varied in glucose fermentation, and did not ferment several other sugars. On the basis of the characteristics of more strains placed in the species it is evident that considerable variation can occur between strains (21, 52, 74). Strains vary in visible production of the yellow pigment, the ability to ferment a few carbohydrates, growth at 45 C, the Voges-Proskauer reaction, and production of hydrogen, carbon dioxide, and ethanol. The species appears to be constant in being iodophilic; growing at 30 to 32 and 37 to 39 C but not at 22 or 48 C; not producing indole or hydrogen sulfide; not reducing nitrate or liquefying gelatin; producing reducing sugars when grown in excess cellulose; producing succinic and acetic acids and at least traces of formic and lactic acids from carbohydrate; and in failing to ferment many carbohydrates. Strains studied by Hungate (74) all produced at least traces of hydrogen but this gas could not be detected in any of the strains of Bryant *et al.* (21). The latter workers found that all strains produced chains under a standard set of conditions and all fermented xylan in addition to cellobiose and cellulose.

A second species, *Ruminococcus albus* Hungate (74), was established to include the strains of ruminococci that did not produce succinic acid. The characteristics of this species were quite variable but most strains differed from the type species in producing little or no yellow pigment; cells were usually arranged singly or in pairs, were gram-negative to gram-variable, and usually not iodophilic. Fermentation products included hydrogen, carbon dioxide, ethanol, acetic acid, formic acid, and lactic acid in various combinations and proportions. Usually, more hydrogen and carbon dioxide were produced than by *R. flavefaciens*. Seven of 14 strains of ruminococci studied by Bryant *et al.* (21) formed a distinct group that differed from *R. flavefaciens* in producing little or no succinic acid, a large amount of ethanol and, with the exception of one strain, gas that included hydrogen. Also, they usually occurred singly and in pairs rather than in chains. They were placed in the species *R. albus* Hungate. They tended to differ from the strains of Hungate, because some produced considerable yellow pigment, all were iodophilic, none produced large amounts of lactic acid, and some strains fermented glucose. Most of the strains of Bryant *et al.* (21) fermented xylan.

Even with the descriptions allowing for considerable variation in characteristics in the two species of ruminococci, some strains could not be placed in either species (21, 74).

Hungate (74) indicated that cellulose digestion should not be regarded as a distinctive character of ruminococci. Bryant and Burkey (12) isolated a few strains that appeared to be identical with the cellulolytic cocci in the few characteristics studied, except that cellulose was not visibly attacked. Later, noncellulolytic cocci having the characteristics of *R. albus* were isolated from the rumen of calves and a mature cow (20). They differed from cellulolytic strains of the species only in fermenting a larger number of carbohydrates than most cellulolytic strains. However, mannitol was the only substance fermented by some (2 of 9 strains) that has not been shown to be fermented by some cellulolytic strains.

A study of nutritional requirements of 2 strains of *R. albus* and 3 strains *R. flavefaciens* indicated a close relationship in that all required or were greatly stimulated by volatile acids from rumen fluid and by isovaleric acid when grown in a medium containing B-vitamins, minerals, casein hydrolysate, Tween 80, cellobiose, and cysteine (1). However, the growth of a strain of *R. albus* investigated by Fletcher (36) was not stimulated

by volatile fatty acids but it required factors from rumen fluid that appeared to be yellow colored, nonvolatile, carboxylic acids with neutralization equivalents of about 223. These results indicate that individual strains of *R. albus* may have different growth requirements.

c. Veillonellae. Using a lactate medium, Johns (81) regularly isolated *Veillonella alcalescens* (*V. gazogenes*) from the rumen of sheep. The numbers found, 1 to 7.4 $\times$ 10^6 per ml, suggested that the organism was only of moderate importance in the rumen of sheep fed on winter grass. Johns indicated that they probably function in lactate fermentation and also appear to be concerned in decarboxylation of succinate to propionate and carbon dioxide. The pH optimum of this reaction for washed cells of the veillonellae was similar to that of washed bacterial cells from rumen fluid. Guiterrez (48) isolated these bacteria from the rumen of sheep in numbers of 1.8 to 5.2 $\times$ 10^5 per ml and from cattle in numbers of 60 to 2000 per ml. Elsden *et al.* (34) also isolated them from sheep.

IV. CONCLUSION

It is evident that the rumen contains a great variety of bacterial species, many of which are present in large numbers in one animal held under one set of conditions. It seems probable that most of the more significant groups have been isolated and that future studies will not disclose nearly as many new groups as have been found in the last decade. Groups of bacteria corresponding to definite species are sometimes easily identified. However, in many cases groups corresponding to genera or subgenera are easily identified, *e.g.*, the genera *Butyrivibrio* and *Ruminococcus* and the succinic acid-producing *Bacteroides*, but the great variability in characteristics between strains in these groups suggests that species specific patterns will be very difficult to find. The species will probably have to be defined to include many variable characteristics or the number of species will become unmanageable.

The attempts that have been made to classify and name ruminal bacteria also show the paucity of information available on the classification of nonsporeforming anaerobes in general. It has been impossible to identify many strains with previously described species from other sources, possibly because they represent species not found in habitats other than the rumen. However, it has been shown that some species of rumen bacteria are common to other habitats and it seems possible that many other species are present in other habitats, such as the oral cavity and intestinal tract of nonruminants, but they either have not been isolated or the descriptions given are too poor to allow identification with rumen organisms.

Although there is still much disagreement as to the relative importance of many species of bacteria isolated from the rumen and it is unlikely that many of these disagreements will be resolved in the near future, it is evident that many species present in large numbers and concerned with functions of great importance in ruminal fermentation have been described. Further studies on the metabolism and grown requirements of these species in pure culture and possibly in known mixtures, along with quantitative studies attempting to correlate the species present with specific reactions in the total ruminal fermentation, will lead to information of value to the animal husbandman and to an understanding of microbial ecology.

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